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Biochemical properties and comparative pharmacology of a coagulant from *Deinagkistrodon acutus* snake venom

Song-Shan Tang [a,*], Xiao-Hua Wang [b,1], Juan-Hui Zhang [c], Bo-Shan Tang [d], Li Qian [e], Pei-Ying Li [a], Lie-Wei Luo [a]

[a] Guangdong Key Laboratory of Pharmaceutical Bioactive Substances, School of Basic Courses, Guangdong Pharmaceutical University, Guangzhou 510006, China
[b] Department of Biochemistry, Guangzhou Medical College, Guangzhou 510009, China
[c] Department of Obstetrics & Gynecology, Guangdong Provincial Corps Hospital, Guangzhou 510507, China
[d] Department of Hematology, Zhujiang Hospital, Southern Medical University, Guangzhou 510280, China
[e] Department of Biochemistry, China Pharmaceutical University, Nanjing 210009, China

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ABSTRACT

A number of snake venom thrombin-like enzymes (TLEs) have already been characterized. Some TLEs play significant roles in vessel injury hemostasis. A novel TLE (*Agacutase*) was purified from *Deinagkistrodon acutus* snake venom by the means of Sephadex G-75, DEAE-Sepharose FF, and Sephadex G-25 column chromatography. Structural analysis indicated that *Agacutase* is a single-chain glycoprotein with a molecular mass of 31,084 Da, isoelectric point of 4.38, optimal activity at 37 °C and pH 6.6, sugar content of 7.6%. Its N-terminal 44 amino acid sequence was determined to be VIGGNECDTNEHRFLAAFFTSRPWIFQCAGTLIHEEWVLAAAHC, showing maximum identity of 80% with that of *Dav-X* protease. The *Agacutase*-induced clotting activity was not influenced by heparin, hirudin, or Dextran 40, but activated by Ca^{2+} and inhibited by PMSF or lactose, which suggests that *Agacutase* is a serine protease and the coagulation activity is independent of *Thrombin*. *Agacutase* with arginine esterase activity specifically cleaves the α-chain of fibrinogen. *Agacutase iv* (0.03–0.12 U/kg) shortened 16–68% of the rabbit blood clotting time. No significant influence was indicated on platelet, Factor II and XIII, or fibrinolytic system. It converts fibrinogen into the soluble fibrin that accelerates hemostasis at wound. Pharmacological comparison showed the hemostatic effect of *Agacutase* lasted 24 h while *Reptilase* did 8 h. Its maximum tolerated, abnormal toxicity, allergic, and hemorrhagin doses were 80 U/kg, 1 U, 2 U, and 50 U, respectively, whereas those of *Reptilase* or *Agacutin* were 35 U/kg, 0.25 U, 0.25 U, and 0.2 U, respectively. The results indicated that *Agacutase* may be a predominant coagulant.

1. Introduction

Snake venom contains various components that affect the mammalian hemostatic system as either procoagulants or anticoagulants (Braud et al., 2000; Matsui et al., 2000). These components interact with diverse proteins of the blood coagulation cascade and the fibrinolytic pathway. Some of the components are serine proteases which are partially similar to *Thrombin* in structure and function. So the enzymes are named snake venom TLEs.

TLEs are widely distributed in the venoms of several genera, *Deinagkistrodon*, *Bothrops*, *Crotalus*, *Lachesis*, *Trimeresurus*, *Ovophis*, and *Gloydius*. Since the coagulation studies on *Reptilase* were reported in 1957, over 100 TLEs have been isolated and sequenced. Current studies show that TLEs can affect many steps in the hemostatic system ranging from activation of coagulation factors to induction of platelet aggregation (Santos et al., 2000; Teng and Ko, 1998). Some TLEs were also found to have biological activity in nervous and complement systems (Wu et al., 2001; Yamamoto et al., 2002). TLEs belong to the trypsin/kallikrein family (Itoh et al., 1988). Although most TLEs only cleave α-subunit of fibrinogen to produce fibrinopeptide A (FPA), there are several TLEs that were shown to cleave α-and/or β-subunits of fibrinogen to produce fibrinopeptides A and/or B (FPB) (Kornalik, 1990). Parenteral administration of TLE makes fibrinogen convert into fibrin. If the conversion occurs rapidly and widely, it can cause anticoagulation. *Thrombin* (Factor IIa) activates various blood coagulation proteins and cells, such as factors V, VIII, XIII, and platelets, endothelial cells, smooth muscle cells which closely relate to hemostatic system, whereas some TLEs have no effects on these factors or cells. Although TLEs can accelerate the clotting of plasma or fibrinogen

* Corresponding author. Address: 280 East Waihuan Road, Guangzhou Higher Education Mega Center, Guangzhou City, Guangdong Province 510006, China. Tel.: +86 20 39352192; fax: +86 20 39352187.
E-mail address: songstang@gmail.com (S.-S. Tang).
[1] Co-first author

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solution *in vitro*, their effects are different *in vivo*. So some TLEs have been developed into clinical drugs.

Since 1950s, some coagulants exerting on fibrinogen, i.e. *Reptilase*, *Hemocoagulase*, and *Agacutin*, have been discovered. *Reptilase* from *Bothrops jararaca* snake venom is an example of successful clinical use of a coagulant. It can obviously shorten the blood clotting and bleeding time *in vivo* so that it has been used widely in the treatment of wound hemorrhages and in the surgery to prevent bleeding. *Hemocoagulase* from *Deinagkistrodon blomhoffii ussurensis* snake venom is single chain TLE with a molecular mass of 35.5 kDa. It has the same N-terminal 15 amino acid residue sequence and similar effect on hemostasis with *Reptilase* (Mu et al., 2005). *Agacutin* from *Deinagkistrodon acutus* snake venom is a heterodimer with 15 and 16 kDa subunits. It *iv* (0.01–0.05 U/kg) shortened the blood clotting and bleeding time to cause better effect on hemostasis than *Reptilase in vivo* (Tang et al., 2009).

In this study, *Agacutase* is another novel coagulant from snake venom. It showed longer effect on hemostasis and far lower toxicity compared with *Reptilase* or *Agacutin*. The purification, structure, biochemical properties, hemostatic mechanism, and toxicities of *Agacutase* are reported in this paper.

2. Materials and methods

2.1. Purification of Agacutase

A 20 g of the crude *D. acutus* snake venom powder (purchased from *Xinyuan Snake Venom Co.*, China) was dissolved in 200 ml of phosphate buffer (0.01 M, pH 6.8) and centrifuged at 6000 rpm for 20 min. 50 ml of the supernatant (4750 mg protein) was applied to a gel filtration column (7 × 100 cm) of Sephadex G-75 and eluted with 0.01 M phosphate buffer (pH 6.8) and flow rate of 7 ml/min. The active fraction was collected. After all the supernatants were disposed in the G-75 column, all the active fraction from the G-75 column was applied to a DEAE-Sepharose Fast Flow ion exchange column (3 × 20 cm) and eluted with a linear gradient of 0–0.05 M NaCl in 0.01 M phosphate buffer (pH 6.8) and flow rate of 15 ml/min (the same flow rate below). After dialyzed against 0.01 M phosphate buffer (pH 6.8) for 24 h, the active fraction was re-applied to the regenerated DEAE-Sepharose FF column and eluted with a linear gradient elution of 0.025–0.05 M NaCl in 0.01 M phosphate buffer (pH 6.8). After dialyzed against 0.01 M phosphate buffer (pH 7.8) for 24 h, the active fraction was applied to the regenerated DEAE-Sepharose FF column for the third time and eluted with a linear gradient elution of 0.025–0.05 M NaCl in 0.01 M phosphate buffer (pH 7.8). Before the pure *Agacutase* was lyophilized, the active fraction was applied to a Sephadex G-25 column (7 × 100 cm) for desalting using cold water (4 °C) as eluent and flow rate of 7 ml/min. The active fractions in the column chromatographs were determined according to the results of human plasma clotting activity and SDS–PAGE. One vial for the animal experiments contained 1 unit of *Agacutase* and 1% Dextran 40 (purchased from the State Food Drug Administration, SFDA). The preparative liquid chromatographer (Assembly of HS-21-1 UV detector, BT-22B Peristaltic Pump, TH2000 Gradient Generator, and 3057 Recorder; *Shanghai Qingpu-Huxi Instruments Factory*) was used in the preparation procedure. The absorbance of the column effluent was monitored at 280 nm.

2.2. Structural analysis

High performance liquid chromatographer (HPLC) (*Shimadzu* LC-10A, Japan) was performed to analyze the purity of *Agacutase* by using gel-filtration HPLC column (SEC-S 2000, *Phenomenx Co.*), an eluent of 0.2 M phosphate buffer (pH 6.8), and a flow rate of 1 ml/min. SDS–PAGE (12% gel concentration) was performed by loading 10–60 μg of *Agacutase* sample, control sample *Acutin* (41 kDa) and protein molecular mass standards (14.4–97.4 kDa) (Laemmli, 1970). The accurate molecular mass was obtained by mixing 0.75 μl of *Agacutase* (200 ng/μl) into saturated sinapic acid and applying to a mass spectrometer (MS) Biflex III (*Bruker Daltonics Co.*, USA). The pure *Agacutase* was blotted on PVDF membrane (*Minipore Co.*) and the corresponding band on the PVDF membrane was excised for sequence analysis via the Edman degradation method (ABI Procise 491 Protein Sequencer, *ABI Co.*, USA). An isoelectric focusing (IEF) electrophoresis was performed on a Sanhen electrophoresis apparatus (ECP3000, *Beijing Medical Apparatus Co.*) by using PAG gel matrix (7% gel concentration) and Ampholine (pH 3.5–9.5, *Pharmacia Co.*, Sweden). The pH values which were measured at each 0.5 cm region of the gel were plotted against the corresponding gel lengths and the resulting isoelectric point (pI) of *Agacutase* was calculated. 0.2 ml of *Agacutase* solution (50 U/ml), 0.2 ml of phenol solution (5%, w/v), and 1.8 ml of concentrated sulfuric acid were mixed for 20 min. The sugar content was obtained by using colorimeter at A_{490} nm (Zhou et al., 2005).

2.3. Analysis of biochemical properties

The healthy human plasma (*Guangdong Provincial Corps Hospital*), bovine fibrinogen solution, and *Agacutase* solution were pre-incubated individually for 3 min at a definite temperature in a glass incubator. 0.2 ml of plasma was mixed in an equal volume of *Agacutase*-water solution (0.2 U) at 25–50 °C. The optimum temperature of the *Agacutase*-induced clotting activity was obtained by counting the plasma clotting time under the different temperatures. 0.2 ml of plasma was mixed in an equal volume of *Agacutase*-phosphate buffer solution (0.2 U enzyme was dissolved in 0.02 M phosphate buffer, pH 5.5–8.5, respectively) at 37 °C. The optimum pH value of the *Agacutase*-induced clotting activity was obtained by counting the plasma clotting time under the different pH values. 0.2 ml of healthy human plasma was mixed in an equal volume of *Agacutase* water solution (0.2 U) containing sodium heparin of 50–1000 U, phenylmethanesulfonyl fluoride (PMSF) of 1–10 mM, or hirudin of 2.5–50 antithrombin units (AT-U) to determine human plasma clotting time at 37 °C. 0.2 ml of *Agacutase* solution (0.2 U) containing $CaCl_2$ of 0.45–7.2 mM, lactose of 0.06–0.30 M, or Dextran 40 of 0.5–2.0 (g/dl) was mixed with 0.8 ml of bovine fibrinogen water solution (0.4 g/dl, the bovine fibrinogen powder was purchased from SFDA) to determine bovine fibrinogen clotting time at 37 °C. Porcine *Thrombin* (*Sigma Co.*) was required as a control in some experiments. In order to observe whether the *Agacutase*-induced plasma clot was re-dissolved, the urea solubility assay was performed by adding 1 ml of 3 M urea to a human plasma clot previously prepared.

2.4. Determination of enzyme activity

The enzyme activity of *Agacutase* was assessed according to the *Reptilase* Unit (KU) method (Kornalik, 1985). In brief, after 0.2 ml of human plasma or 0.8 ml of bovine fibrinogen solution (0.4 g/dl) pre-incubated for 3 min was mixed with 0.2 ml of *Agacutase* at 37 °C, the human plasma or bovine fibrinogen clotting time was obtained by counting the time from the mixture to the initial cloudiness. The KU was used *in vivo* and *in vitro* experiments. In the coagulation assay, each sample was assayed three times and the means were recorded as results. Arginine esterase activity was determined using the BAEE (N-benzoyl-L-arginine ethyl ester hydrochloride) method (Wei and Chen, 2004). Alkaline phospho-

tase, L-amino acid oxidase, or phospholipase A_2 activity was assayed to examine if *Agacutase* presents other enzyme activities (Tang et al., 2009).

2.5. Enzyme hydrolysis of fibrinogen

2.5.1. Analysis of fibrin subunit

Equal volumes of bovine fibrinogen (1 mg/ml) and *Agacutase* (1 U/10 μl) in 50 mM Tris–HCl (pH 6.6) were mixed and incubated at 37 °C for 1, 2, 3, 4 h, respectively. The incubation samples were centrifuged in 10,000 RPM for 5 min. The fibrin subunits in the precipitates were analyzed in SDS–PAGE method (8% gel concentration) and the fibrinopeptides in the supernatants were analyzed in ELISA method. In the experiment porcine thrombin (1 U/100 μl) was used as control.

2.5.2. ELISA analyses of fibrinopeptides

The assays are referred to the company instruction. In brief, 50 μl of standard or sample solution was added in the reaction wells. After 50 μl of diluted biotinylated anti-FPA or FPB antibody was added in the wells, the plate was covered and incubated for 1 h at 37 °C. The cover on the plate was removed and each well was washed in 0.3 ml of washing solution while softly shaking 30 s. After this wash was repeated three times, 60 μl of streptavidin-HRP solution was added to all the wells. After the plate was covered and incubated for 30 min at 37 °C, the wells on the plate was washed like the above procedure. 50 μl of Substrate A and B was added to each well and the plate was incubated for 10 min at 37 °C. Finally after 50 μl of Stop Solution was added to each well, the plate was read in a micrometer plate reader within 30 min at A_{450} nm. In the experiment, 0, 2, 4, 6, 8, 10 ng/ml of the Fibrinopeptide A or B were used as standard.

2.6. Animals and experiments

All animals used (New Zealand white rabbits of 2.3–2.8 kg, Kunming mice of 18–22 g, guinea pigs of 300–400 g; half male and half female) were purchased from Southern Medical University. After raised for 2 days, animals were randomly grouped based on their body weights. In the toxicity evaluations, *Agacutin* and *Reptilase* were used as control and the doses of *Reptilase* were referred to *the Quality Control Standards of Reptilase Vials* from the SFDA.

2.7. Effect on the hemostatic system

The rabbits were classified into 4 groups [*Agacutase* 0.03, 0.06, 0.12 U/kg (Rat No. 20100113) and *Reptilase* 0.06 U/kg (1 KU/vial, Rat No. 786808, *Basle Co*., Sweden)]. Enzyme solution (1 U/ml, prepared with 0.9% sodium chloride) was injected in the rabbit ear marginal vein in a volume of 0.2 ml/kg. After respiratory anesthesia with medical ether, whole blood samples were collected from rabbit hearts before administration and 10 min, 2 h, 4 h, 8 h, and 24 h after *iv*. The Lee-White method (Yamashita et al., 1997) was used in the whole blood clotting time assay (CT). The blood fibrinogen concentration (FIB), activated partial thrombin time (APTT), prothrombin time (PT), and thrombin time (TT) were measured with a 4-Channel Coagulation Analyzer C2004 (*Beijing Puli Biochemical Instrument Co*., *Ltd*, China) and STA Kit (France). Platelet counting was performed with a CELL-DYN3599R apparatus (*Abbot Laboratories*, USA). The platelet activating activity was obtained by determining TXB_2 concentration according to the TXB_2 RIA kit protocol (Medical Department of Suzhou University) with FJ-2003/50 γ-radioamounter (*Shanghai Radioelectrical Co*. China). NJ4 aggregation-meter and LBY-N6C viscosity-meter (*Beijing Puli Biochemical Instrument Co*., *Ltd*, China) were used in the platelet aggregation and blood viscosity assay. The euglobulin clotting time (ECT) and euglobulin lysis time (ELT) were determined according to Xu et al. (1994).

2.8. Toxicity evaluations

The median lethal dose (LD_{50}) and the maximum tolerated dose (MTD) for mice were assessed. The MTD assay was performed in 12 mice by intravenous injecting a 0.5 ml volume containing maximum dose of *Agacutase* once and following 14-day observation. After five mice of 18–22 g were injected subcutaneously in the back with 0.2 ml of *Agacutase* (50 U/0.2 ml, diluted in 0.9% NaCl), the animals were sacrificed and stripped for hemorrhage check after 24 h. According to the Chinese Pharmacopoeia II (2010 version), 0.5 ml of *Agacutase*-NaCl solution containing 0.5, 1.0, or 1.5 U (prepared in 0.9% of NaCl) was respectively injected *iv* in 10 mice to observe its abnormal toxicity. 54 guinea pigs were randomly classified into 9 groups. They were primed *ip* with varying sensitizing doses of *Agacutase* (0.5, 1.0, 2.0, 4.0 U/kg), or *Reptilase* (0.25, 0.5 U/kg), *Agacutin* (0.125, 0.25 U/kg), BSA (1 mg/kg) on days 1, 3, 5, and were challenged *iv* with the doubling sensitizing doses on day 14 or 21. In the experiment, 0.9% NaCl and BSA were used as control. The allergic symposia, i.e. sternutation, nosegrab, diarrhea, vomiting, and bronchial asthma et al., were recorded in 30 min.

2.9. Statistical methods

The statistical significance between the experimental groups was evaluated by Variance and Dunnett's *t* test, using SAS 9.0 statistical software.

3. Results

3.1. Purification of Agacutase

Agacutase was purified with serial column chromatographs: Sephadex G-75 column (Fig. 1A), 1st, 2nd, 3rd DEAE-Sepharose FF column (Fig. 1B–D), and Sephadex G-25 desalting column. As summarized in Table 1, the final weight yield and specific activity of *Agacutase* were approximately 0.028% and 580.4 U/mg. The overall maximum purification was about 3527 folds.

3.2. Purity and structure of Agacutase

The results of the gel-filtration HPLC (Fig. 2A) and SDS–PAGE (Fig. 2B) indicated that *Agacutase* was purified to high homogeneity. The relative molecular mass (MW) of *Agacutase* in the SDS–PAGE method is about 37 kDa and the accurate MW in the MALDI-TOF MS method was 31,084 Da (Fig. 3). *Agacutase* contains 7.6% of sugar content (w/w). The N-terminal 44 amino acid residues of *Agacutase* were determined to be VIGGNECDTNEHRFLAAFFTSRPWIFQCA GTLIHEEWVLAAAHC. A BLAST analysis of this sequence revealed 80% maximum identity (35/44) with that of *Dav-X* or *Thrombin-Like protein* 3 protease from *D. acutus* snake venom, 59% identity (26/44) with that of *Acutobin*, 39% identity (17/44) with that of *Acubin*, and 34% identity (15/44) with that of *Bothrombin* (*Reptilase*) from *Bothrops jararaca* (Fig. 4). A pI value of 4.38 was obtained according to the relationship between the gel pH gradient and the gel length (Fig. 5).

3.3. Biochemical properties of Agacutase

According to the clotting activity *vs* temperature (Fig. 6A) and clotting activity *vs* pH (Fig. 6B) curves, the optimal temperature and optimal pH values of *Agacutase* were determined to be 37 °C

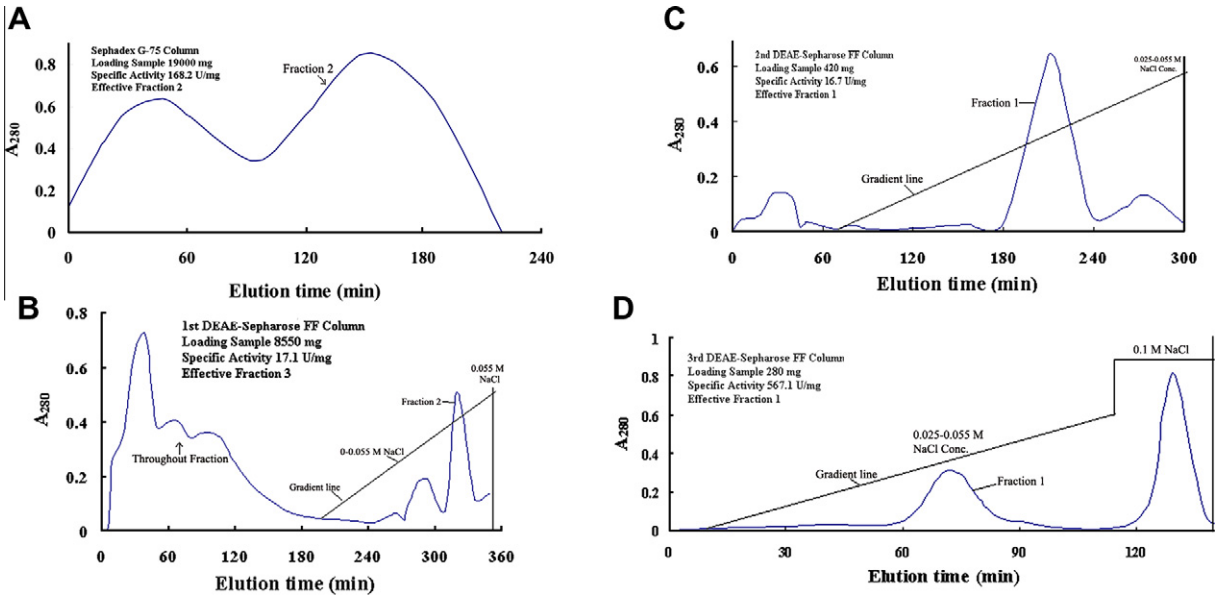

Fig. 1. (A) Sephadex G-75 gel filtration column chromatography of the crude venom. A column of 7 × 100 cm and flow rate of 7 ml/min were used. (B, C, and D) DEAE-Sepharose FF column chromatography. A column of 3 × 20 cm and flow rate of 15 ml/min were used. Throughout fraction: the fraction before gradient.

Table 1
Summary of purification procedure of *Agacutase*.

Purification steps	Protein weight (mg)	Activity units (U)[a]	Specific activity (U/mg)	Weight yield (%)	Purification fold[b]
Crude venom powder	20,000	–	–	100	1
Pre-treatment of venom	19,000	1,600,000	84.2	95	1.05
G-75	8550	1,440,000	168.4	42.8	2.34
1st DEAE-Sepharose F.F	420	7200	17.1	2.1	48
2nd DEAE-Sepharose F.F.	280	4680	16.7	1.4	71
3rd DEAE-Sepharose F.F.	7	3970	567.1	0.035	2857
G-25	5.67	3291	580.4	0.028	3527

[a] Activity (KU) was determined by using human plasma and bovine fibrinogen as the substrates.
[b] Purification fold was determined by using protein weight and protein concentration was determined with the Lowry method.

and 6.6, respectively. 20–120 AT-U/ml of hirudin (Fig. 7A) or 50–1000 U of heparin (Fig. 7B) had no significant influence on the *Agacutase*-induced plasma clotting activity, whereas the *Thrombin*-induced plasma clotting activity was obviously inhibited by more than 50 U of heparin. PMSF significantly inhibited the *Agacutase*-induced plasma clotting activity (Fig. 7C). The *Agacutase*-induced bovine fibrinogen clotting activity was gradually stimulated with the increase of calcium concentration (Fig. 8). When 1 ml of Urea solution (3 M) was added to human plasma clot previously formed by *Agacutase* and the clot was quickly re-dissolved with slightly shaking.

3.4. Enzyme activities and hydrolysis on bovine fibrinogen

Agacutase displayed significant human plasma or fibrinogen clotting activity in our experiment, but it did not present alkaline phosphotase, L-amino acid oxidase, or phospholipase A_2 activity. 0.2 ml of *Agacutase* water solution (1 U/ml) coagulates both 0.2 ml of healthy human plasma at 37 °C in 60 ± 20 s and 0.8 ml of bovine fibrinogen solution (0.4%) in 133 ± 20 s. Arginine esterase activity assay showed that 1 mg of the enzyme hydrolyzed 140.8 μmol of BAEE per minute, or 1 U of *Agacutase* hydrolyzed 0.2416 μmol of BAEE per minute, indicating that *Agacutase* is an arginine esterase with weak BAEE activity. *Agacutase* only hydrolyzed α-subunit of bovine fibrinogen to release FPA, whereas porcine thrombin exerted on both α and β subunits of fibrinogen to release FPA and FPB (Fig. 9A–C).

3.5. Influence on the rabbit blood system

3.5.1. CT assay

Compared with the data before *iv*, *Agacutase* obviously shortened the rabbit blood clotting time in a dose-dependent manner. The initial effect was in 10 min and peak value appeared at 2 h. The hemostatic effect of *Agacutase* lasted for 24 h, whereas that of *Reptilase* did for 8 h (Table 2).

3.5.2. FIB assay

FIB for each experimental group showed a trend of gradual decrease after *iv*, but there was no significant difference in group and between groups (Table 3).

3.5.3. APTT assay

Compared with *Reptilase*, the APTT results of *Agacutase* did not indicate significant difference in statistics after *iv* (Table 4).

3.5.4. Blood viscosity assay

The blood viscosities under the same pressure (10, 60, or 150 s^{-1}) decreased gradually (Table 5). They were appropriate for the results of CT and FIB.

3.6. Toxicity results

3.6.1. Maximum tolerated dose (MTD)

The median lethal dose (LD_{50}) of *Agacutase* was not obtained because of a minimal toxicity data. via 14-day continuous observa-

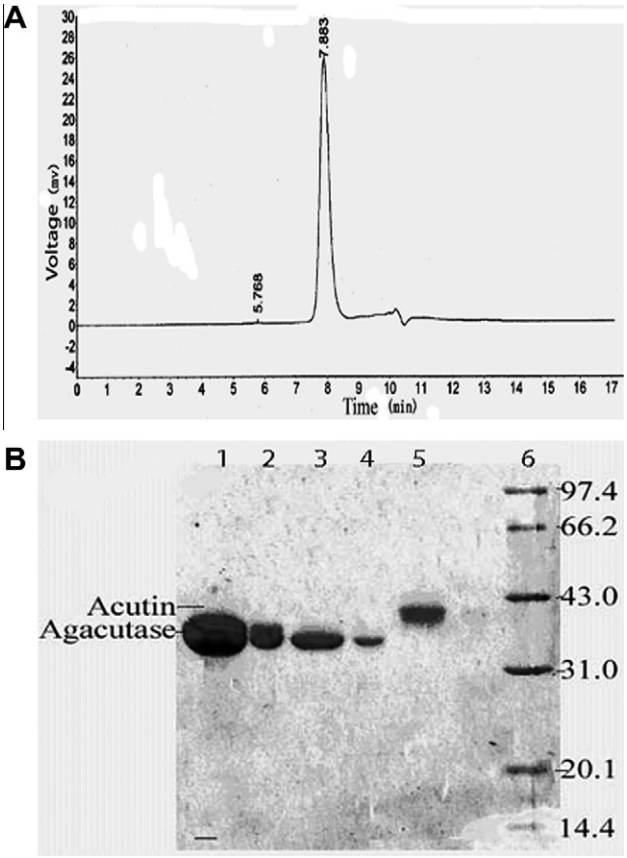

Fig. 2. (A) Gel-filtration HPLC analysis. (B) SDS–PAGE (12%) analysis of *Agacutase*. Lane 1–4: 60, 20, 30, 10 μg sample loaded; Lane 5: *Acutin* (41 kDa); Lane 6: Protein molecular mass standards (14.4–97.4 kDa).

tion after *iv*, the maximum tolerated dose of *Agacutase* in mice was determined to be 80 U/kg, whereas that of *Reptilase* or *Agacutin* was 35 U/kg (Table 6). During the observation period, no abnormal observations were discovered regarding the behavior, diet, fur color, and death of the mice.

3.6.2. Abnormal toxicity

0.5, 1.0, or 1.5 U of *Agacutase* in 0.5 ml volume was injected *iv* in 10 mice, respectively. The doses of 0.5 and 1.0 U did not cause death in 24 h, 1.5 U of *Agacutase* leaded to 10% death rate. Hence, the maximum negative abnormal toxicity dose of *Agacutase* was determined to be 1.0 U/0.5 ml, whereas that of *Reptilase* or *Agacutin* was 0.25 U/0.5 ml (Table 7).

3.6.3. Hemorrhagin assay

0.2 ml (50 U) of *Agacutase* solution (430.7 μg/ml) did not cause bleeding reaction at the injection point, whereas the equivalent dose of *Agacutin* was 0.2 U/0.2 ml (50 μg/ml).

3.6.4. Allergic test

The results showed that no more than 2.0 U/kg of *Agacutase*, or 0.25 U/kg of *Reptilase*, or 0.125 U/kg of *Agacutin* did not cause allergic reaction, whereas the BSA group had 100% positive reaction rate (Table 8).

4. Discussion

Since the 1970s, many snake venom enzymes, such as *Ancrod*, *Batroxobin*, *Crotalase*, *Acutin*, *Reptilase*, *Hemocoagulase*, and *Bothropase*, have been developed into clinical drugs. These drugs play definite roles in blood anticoagulation and hemostasis. Among these snake venom enzymes, *Reptilase*, due to the greater efficacy and lower toxicity in clinic, has been used widely as clinical coagulant.

Agacutase with one single chain of 31,084 Da (MW) and 4.38 (pI) presents 37-kDa motility on SDS–PAGE. Maybe its high sugar content of 7.6% is involved in this discrepancy. The structure of *Agacutase* is obviously different from *Reptilase* (32-kDa-single-chain), *Batroxobin* (36-kDa-single-chain), or *Agacutin* (31-kDa-heterodimer). The unique N-terminal 44 amino acid sequence suggests that *Agacutase* is a novel snake venom TLE and the BLAST results of the N-terminal sequence showed extremely low identity with that of *Reptilase* or *Agacutin*.

The purification results indicated that the final weight yield of *Agacutase* was 0.028%. We obtained 7 mg of *Agacutase* from 20,000 mg of the venom powder, which suggests that *Agacutase* is a minor component in the snake venom. As shown in Table 1, other TLE impurities, PBS, and NaCl salt obviously interfered with the determination of *Agacutase* activity, which may explain the non-proportional increase of the specific activities. Sephadex G-25 column was used to desalt the enzyme solution so that the actual activity of the pure enzyme could be determined.

The *Agacutase*-induced clotting activity was not influenced by heparin or hirudin, but activated by Ca^{2+} and inhibited by PMSF, which suggests that *Agacutase* is a serine protease like *Reptilase*. In the experiment we found that lactose obviously inhibited the *Agacutase*-induced plasma clotting activity and Dextran 40 hardly influenced the activity (data not shown), which suggest that small molecular lactose may interfere with the recognition between *Agacutase* and fibrinogen molecules, whereas Dextran 40 with larger molecular skeleton (10–30 kDa) may not influence the interaction. That is why Dextran 40 is selected as a preferred pharmaceutical excipient for snake venom TLE vial.

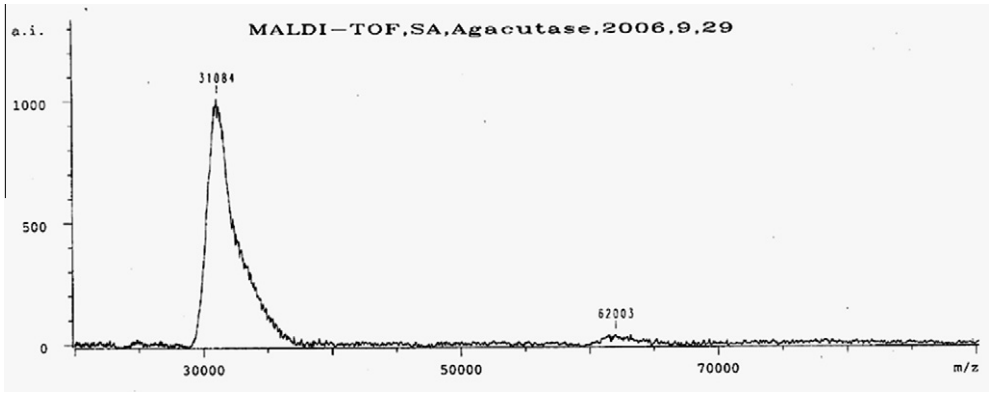

Fig. 3. MALDI-TOF MS analysis of *Agacutase*.

SV enzyme	GI No.	Sequence numbers of N-terminal 1-44 amino acid residues
Agacutase	----------	V I G G N E C D T N E H R F L A A F F T S R P W I F Q C A G T L I H E E W V L A A A H C
Dav-PA	13959649	- - - - - - - - I - - - - - - - V - - - N T T G F F C G G T L I N P E W V V T A - H C D S
Acutobin	13959650	- - - - V - - - I - - - - - - - V - L Y E L T S M T - L - G - - - I N Q - - - V T - - - -
Dav-X	13959647	- - - - V - - - I - - - - - - - - - - K Y Q - - T - - - - - - - I - - Q - - - G - - - -
BthaTl precursor	48093528	- - - - D - - - I - - - - - - - - F L Y P G - F F C S G T L I N Q E W V L T - A H C D T I
Acutin	4378029	- - - - D - - - I - - - - - - - V - - - N T T G F F C G G T L I N P E W V V T A - H C D S
Batroxobin	211031	- - - - D - - - I - - - P - - - F M Y Y - P R Y F C G M T L I N Q E W V L T A - H C N R
Ancrod	403078	- - - - D - - N I - - - - - - V - L Y D - T T R N - L - G - V - I - P - - - I T - K - -
Crotalase	250562	- - - - D - - N I - - - - - - V - I Y D
ACC-C	461512	- - - - D - - N I - - - - - - - L V Y A N G S L C G G T L I N Q E W V L T A R H C D R G
Bothrombin	14285806	- - - - D - - - I - - - R - - - F M Y Y - P Q Y F C G M T L I N Q E W V L T A - H C D K
Dav-KN	13959648	- - - - D - - N I - - - P - - V L V Y Y D D Y Q C G G T L L N E E W V L T A A H C N G K
DAV-WY	157849594	- - - - - - - - I - - - - - - V A L Y D N L T G T L D - G - - - I N Q - - - - S - - H C
Thrombin like protein 3	57791683	- - - - V - - - I - - - - - - - - - K Y Q - - T - - - - - - - I - - Q - - - G - - - -
TLE 2	57791760	- - - - D - - - I - - - - - - - - L Y - - E - - T - H - - - - - - I - K Q - - - G - - - -
Defibrase	31322297	- - - - D - - N I - - - - S - V - - - N - T G F F C S G T L V N E E W V L S A - H C D S
Factor V-activating proteinase gamma	134130	- V - - D - - N I - - - P - - V - L Y - - A S S T I H - - - A - I N R - - - - T - - - -

Fig. 4. BLAST alignment of the N-terminal 44 amino acid sequence of *Agacutase*. SV enzyme: snake venom enzyme; GI No: GenInfo Identifier number.

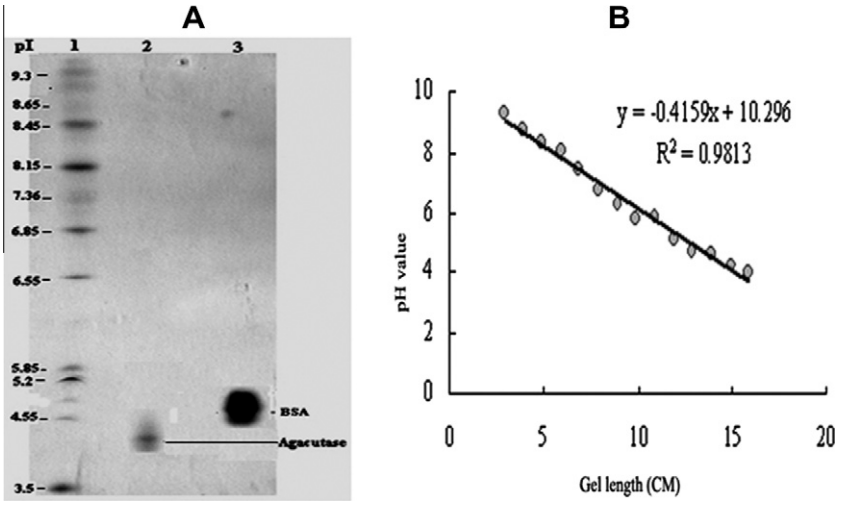

Fig. 5. IEF electrophoresis analysis of *Agacutase*. (A) IEF image. Lane1: IEF markers (pI 3.5–9.3); Lane 2: *Agacutase*. Lane 3: BSA; (B) Relationship curve between pH value and gel length.

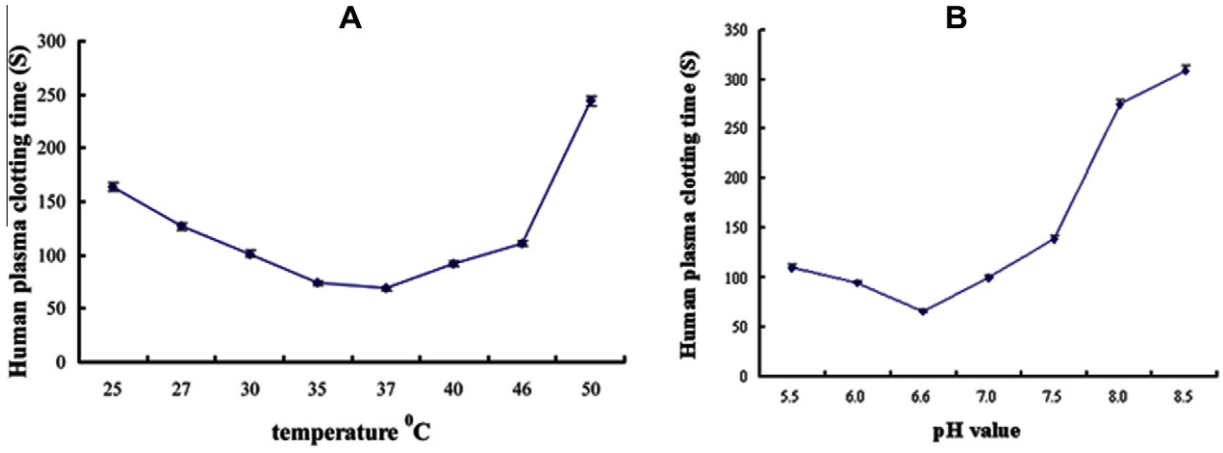

Fig. 6. (A) Clotting activity *vs* temperature curve; (B) Clotting activity *vs* pH curve.

Compared with *Acutin* (*Batroxobin*-like TLE, 3000 BU/mg or 510 NIH units/mg) or *Thrombin* (2000 NIH units/mg), *Agacutase* (580.4 KU/mg or 23.2 NIH units/mg) shows less bovine fibrinogen clotting activity or arginine esterase activity, suggesting that

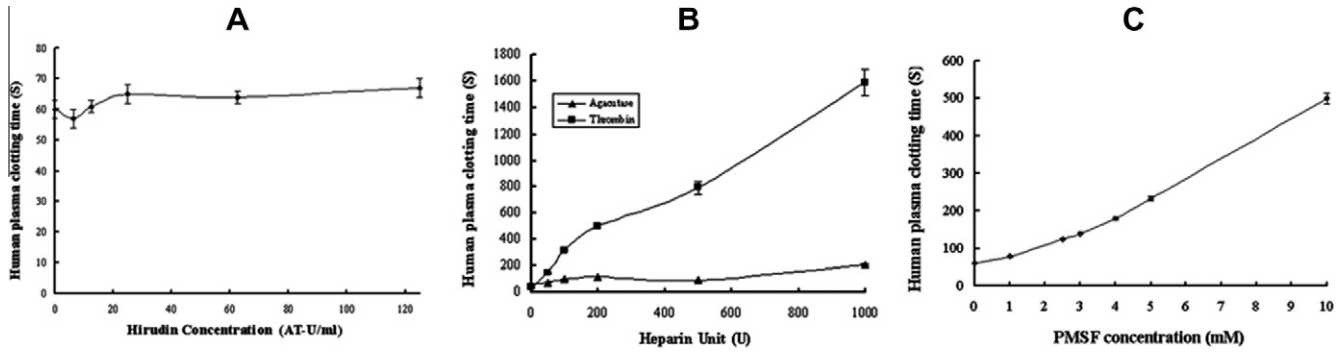

Fig. 7. Influence of hirudin (A), heparin (B), or PMSF (C) on the human plasma clotting activity of *Agacutase*.

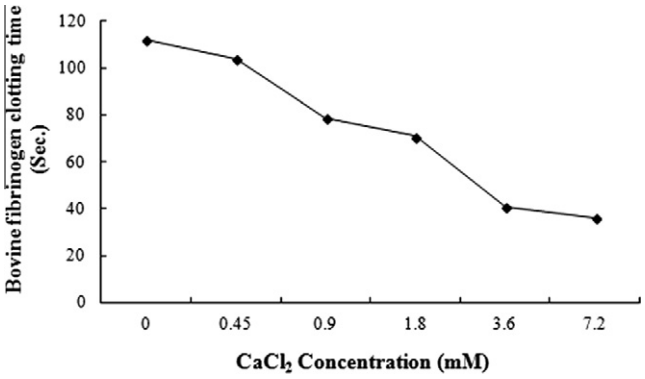

Fig. 8. Influence of Ca^{2+} on the bovine fibrinogen clotting activity of *Agactase*.

Agacutase has a stronger effect on hemostasis rather than an anticoagulant or thrombosis function. Lower affinity of *Agacutase* to fibrinogen induces low level of soluble fibrin in the blood vessel. However, higher affinity of *Batroxobin* or *Acutin* to fibrinogen causes rapid conversion of fibrinogen into soluble fibrin, which subsequently induces endogenous t-PA release to lead to the activation of the fibrinolytic system. The arginine esterase activity and the hydrolysis of bovine fibrinogen show that *Agacutase* cleaves α-chain of fibrinogen like *Agacutin*, whereas *Thrombin* hydrolyzes both α and β-subunits.

One lyophilized vial of *Reptilase* or *Agacutin* containing 1 KU (1 KU = 0.04 NIH Unit) shortens 1/3–1/2 of the adult or rabbit blood clotting time, whereas one vial of *Batroxobin* containing 5 or 10 BU (*Batroxobin* unit, 1 BU = 0.17 NIH unit) delays blood clotting at least 30 min. The *iv* or *im* administration of *Batroxobin* can lead to blood anticoagulation by inducing a rapid and prolonged defibrinogenation. The difference in specific activity between *Agacutase* (580 U/mg) and *Agacutin* (20 U/mg) can also explain the discrepancy in their effects. These properties help us to better understand their inherent differences.

The *in vivo* effect indicated that *Agacutase* (0.03–0.12 U/kg) *iv* shortened 16 to 68% of the rabbit blood clotting time and the hemostatic effect was in a dose-dependent manner without fibrinolytic effect. The significant effect of *Agacutase* lasted for 24 h whereas that of *Reptilase* only for 8 h. Our preliminary experiments verified that no matter whether 0.01, 0.025, 0.06 or 0.12 U/kg of *Reptilase* was administered, it caused no more than 8-h significant

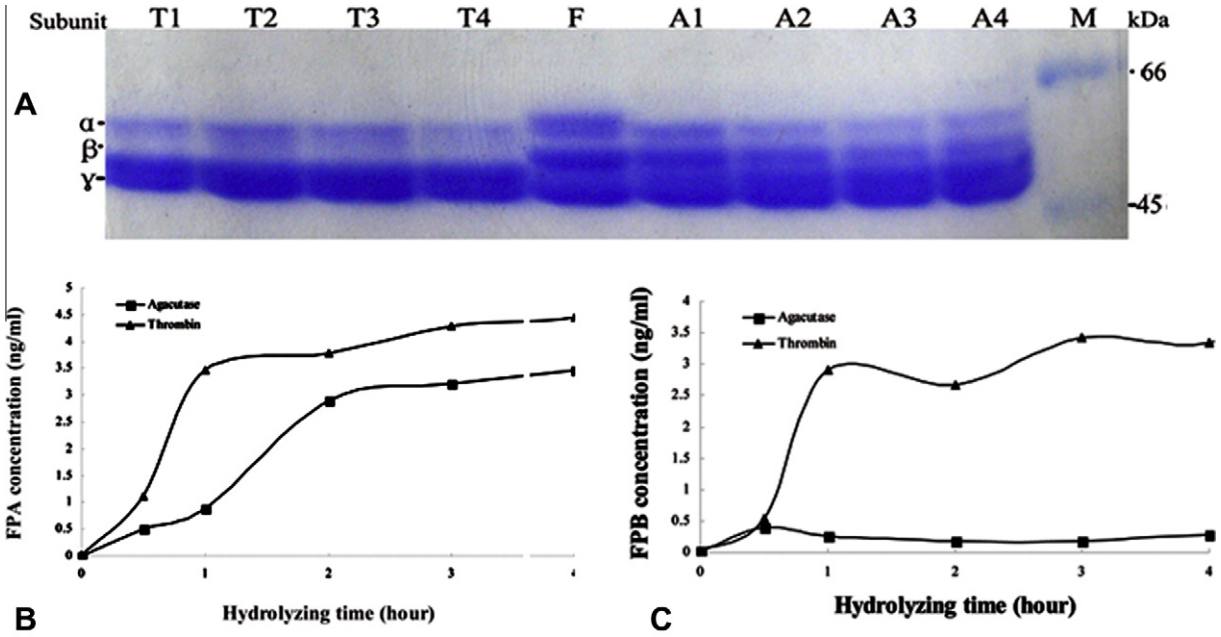

Fig. 9. (A) Hydrolysis analysis of *Agacutase* to bovine fibrinogen. Lane T1–T4: fibrinogen hydrolyzed by porcine *Thrombin* for 1, 2, 3, 4 h; Lane F: bovine fibrinogen (α, β, and γ represent the three subunit bands). Lane A1–A4: fibrinogen hydrolyzed by *Agacutase* for 1, 2, 3, 4 h; Lane M: protein molecular mass standards (14.4–94 kDa); (B) ELISA analysis of FPA concentration; (C) ELISA analysis of FPB concentration. FPA or FPB standard concentrations 0, 2, 4, 6, 8, 10 ng/ml.

Table 2
Influence of *Agacutase* on the rabbit whole blood clotting time (s) ($\bar{x} \pm s$, $n = 6$).

Group (U/kg)	Before *iv*	10 min after *iv*	2 h after *iv*	4 h after *iv*	8 h after *iv*	24 h after *iv*	*F* value
Agacutase 0.03	604.6 ± 78.3	411.9 ± 72.3*	299.9 ± 89.6*	330.4 ± 61.2*	561.3 ± 101.1	526.7 ± 78.0	17.12
Agacutase 0.06	624.6 ± 99.1	259.0 ± 90.6*	243.4 ± 41.3*	345.9 ± 89.2*	398.8 ± 155.1*	483.1 ± 85.2	11.98
Agacutase 0.12	661.5 ± 89.3	201.6 ± 58.3*	251.9 ± 68.4*	301.2 ± 89.8*	530.0 ± 36.1*	551.2 ± 45.1*	45.13
Reptilase 0.06	572.6 ± 101.6	420.0 ± 78.8*	204.3 ± 51.2*	375.6 ± 101.4*	289.2 ± 77.2*	573.3 ± 64.0	23.35

* $P < 0.05$ by Dunnett's *t* test *vs* Result before administered.

Table 3
Influence of *Agacutase* on the rabbit blood fibrinogen (g/l) ($\bar{x} \pm s$, $n = 6$).

Group (U/kg)	Before *iv*	10 min after *iv*	2 h after *iv*	4 h after *iv*	8 h after *iv*	24 h after *iv*	*F* value
Agacutase 0.03	3.89 ± 0.59	3.24 ± 0.69	3.53 ± 0.89	2.55 ± 0.71	2.78 ± 0.65	3.65 ± 0.99	2.56
Agacutase 0.06	3.12 ± 0.23	3.30 ± 0.54	2.61 ± 0.42	3.09 ± 0.21	2.32 ± 0.66	3.61 ± 0.71	2.60
Agacutase 0.12	3.41 ± 0.51	2.99 ± 0.76	3.12 ± 0.69	2.91 ± 0.22	2.53 ± 0.61	2.77 ± 0.32	2.24
Reptilase 0.06	3.18 ± 0.32	3.23 ± 0.70	3.01 ± 0.56	3.31 ± 0.77	3.14 ± 0.65	4.11 ± 0.91	2.23

Table 4
Influence of *Agacutase* on the rabbit blood APTT (s) ($\bar{x} \pm s$, $n = 6$).

Group (U/kg)	Before *iv*	10 min after *iv*	2 h after *iv*	4 h after *iv*	8 h after *iv*	24 h after *iv*	*F* value
Agacutase 0.03	39.9 ± 9.1	46.3 ± 9.1	41.1 ± 12.3	36.5 ± 13.1	45.9 ± 13.3	38.8 ± 10.1	0.43
Agacutase 0.06	43.9 ± 12.3	44.3 ± 5.1	26.9 ± 7.7*	39.5 ± 11.2	43.9 ± 6.8	42.1 ± 8.3	7.54
Agacutase 0.12	35.7 ± 3.9	41.8 ± 6.9	33.4 ± 9.0	41.8 ± 7.6	42.4 ± 5.3	42.6 ± 6.2	2.54
Reptilase 0.06	38.7 ± 9.3	32.6 ± 7.2	37.8 ± 7.8	39.8 ± 9.2	43.7 ± 6.6	42.5 ± 10.3	2.89

* $P < 0.05$ by Dunnett's *t* test *vs* Result before administered.

Table 5
Influence of *Agacutase* on the rabbit blood viscosity (%) ($\bar{x} \pm s$, $n = 6$).

Groups	Dose (U/kg)	Blood viscosities under the same pressure (10/60/150 s^{-1})					
		Before *iv*	10 min after *iv*	2 h after *iv*	4 h after *iv*	8 h after *iv*	24 h after *iv*
		5.58 ± 0.59/	5.14 ± 1.09/	5.17 ± 0.87/	4.76 ± 0.51/	3.87 ± 0.82*/	4.56 ± 0.64/
	0.03	3.14 ± 0.19/	3.12 ± 0.33/	2.88 ± 0.38/	2.89 ± 0.34/	2.48 ± 0.33*/	2.68 ± 0.39*/
		2.79 ± 0.18	2.66 ± 0.29	2.44 ± 0.32	2.46 ± 0.28	2.17 ± 0.23*	2.28 ± 0.30*
		5.43 ± 0.58/	5.34 ± 0.49/	4.98 ± 0.49/	4.34 ± 0.48*/	3.49 ± 0.53*/	5.01 ± 1.28/
Agacutase	0.06	3.19 ± 0.11/	3.16 ± 0.14/	2.86 ± 0.13*/	2.84 ± 0.16*/	2.34 ± 0.17*/	2.66 ± 0.26*/
		2.73 ± 0.09	2.68 ± 0.09	2.43 ± 0.09*	2.45 ± 0.13*	2.06 ± 0.12*	2.19 ± 0.20*
		5.39 ± 0.46/	5.75 ± 0.75/	5.14 ± 1.13/	5.02 ± 1.42/	3.84 ± 0.65/	5.18 ± 1.44/
	0.012	3.34 ± 0.24/	3.37 ± 0.33/	3.08 ± 0.26/	3.42 ± 0.99/	2.55 ± 0.29*/	2.84 ± 0.39/
		2.76 ± 0.19	2.79 ± 0.26	2.60 ± 0.19	2.70 ± 0.38	2.22 ± 0.22*	2.29 ± 0.20*
		3.24 ± 0.20/	4.74 ± 0.95/	5.14 ± 0.46/	5.15 ± 0.55/	3.31 ± 0.21*/	4.54 ± 0.36/
Reptilase	000.06	5.24 ± 0.46/	3.04 ± 0.39/	3.01 ± 0.19/	3.01 ± 0.35/	2.45 ± 0.17*/	2.62 ± 0.18*/
		2.78 ± 0.14	2.61 ± 0.31	2.50 ± 0.18*	2.50 ± 0.26*	2.21 ± 0.15*	2.18 ± 0.14*

* $P < 0.05$ by Dunnett's *t* test *vs* Result before administered.

Table 6
Maximum tolerated dose analysis of *Agacutase* for mice.

Group	Administered approach	Dose (U/kg)	Death rate (%)	*n*
Agacutase	*iv*	80	0	12
Agacutin	*iv*	35	0	12
Reptilase	*iv*	35	0	12

Table 7
Abnormal toxicity analysis.

Enzyme	Dose (U/0.5 ml/mouse)	Death number	Death rate (%)	*n*
Agacutase	0.5	0	0	10
	1.0	0	0	10
	1.5	1	10	10
Agacutin	0.25	0	0	10
	0.5	1	10	10
Reptilase	0.25	0	0	10

Table 8
Allergic reaction analysis.

Group	Dose	Response number	Positive response rate (%)	*n*
0.9% NaClL	0.5 ml/guinea pig	0	0	6
BSA	2 mg/ml	6	100	6
Reptilase	0.25 U/kg	0	0	6
	0.50 U/kg	3	50	6
Agacutin	0.125 U/kg	0	0	6
	0.25 U/kg	1	17	6
Agacutase	0.5 U/kg	0	0	6
	1.0 U/kg	0	0	6
	2.0 U/kg	0	0	6
	4.0 U/kg	1	17	6

effect on hemostasis, which showed that *Agacutase* had better effect on hemostasis than *Reptilase*. The longer effect of *Agacutase* may result from a longer half-life due to the higher sugar content as *Agacutin* and the lower fibrinogen clotting activity (1 KU: 133 s) than *Reptilase* (1 KU: 100 s). The lower toxicity may result

from the dosage of less content (one unit of vial contained 1.72 μg of *Agacutase*) and a higher specific activity (580 U/mg). Various assays of blood coagulation parameters suggest that *Agacutase* does not activate pro-thrombin (Factor II) and Fibrin Stabilizing Factor (Factor XIII) (data not shown). The results of the heparin and hirudin assay also indicated that the plasma clotting mechanism of *Agacutase* was different from that of *Thrombin*, but similar to that of *Reptilase* or *Agacutin*. The *Agacutase*-induced coagulation is independent of *Thrombin* activity.

The results of CT, FIB, and blood viscosity indicated that *Agacutase* generated soluble fibrin level in the blood vessel. When wound occurs, the soluble fibrin can accelerate hemostasis at wound. In normal blood vessels the half-life of soluble fibrin is a few minutes. *Agacutase* has no significant impact on platelet count and function, or ECT and ELT, etc. (data not shown).

Since *Agacutase* had very similar *in vitro* and *in vivo* properties to *Reptilase* or *Agacutin*, its activity was determined according to the *Reptilase* unit (KU). The quality control standards of the abnormal toxicity, allergic test, and hemorrhagin test for *Reptilase* or *Agacutin* vial were 0.25 U, 0.25 U, and 0.2 U, whereas for *Agacutase* they were 1 U, 2 U, and 50 U, which suggest that *Agacutase* has far lower toxicity than *Reptilase* or *Agacutin*. In the animal experiments, the doses of *Reptilase* used were referred to *the Quality Control Standard* of *Reptilase Vial* from SFDA.

5. Conclusions

All the experimental results indicate that *Agacutase* may be a predominant coagulant in clinic and it may replace *Reptilase* and *Agacutin*. Further research will profoundly help us to understand *Agacutase*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2013.02.002.

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